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Ethanolic Extract of *Glycyrrhiza glabra* to Ameliorate Oxidative Stress - Studies *In vitro*

Dhanya K Chandrasekharan [1,*], Femi Varghese [1]

[1] Department of Microbiology, St. Mary's College, Thrissur, Kerala, India
* Correspondence: dhanuchandra@gmail.com;

Scopus Author ID 35077534000
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Abstract: Plants are major sources of bioactive organic molecules of interest for the pharmaceutical industry and are being screened for new drugs and chemicals. *Glycyrrhiza glabra* Linn. (Family: Fabaceae) is a very well-known medicinal plant traditionally used as medicine. Its roots and rhizomes are the medicinal parts used and are reported to possess antitumor, antimicrobial, antiviral, anti-inflammatory, immunoregulatory activities. The ethanolic extract of Glycyrrhiza glabra was prepared in the present work, and its phytochemical analysis and HPLC were done. The *in vitro* antioxidant activity assays such as DPPH radical scavenging assay, Hydroxyl radical scavenging assay, total antioxidant activity assay, and total reducing power assay was done, and the results showed significant antioxidant activity. The extract was analyzed further to evaluate the ability to protect against oxidative stress in chicken liver tissue. The extent of lipid peroxidation and glutathione in H_2O_2 and/or *Glycyrrhiza glabra* extract-treated tissue indicated the extract's potential to protect against oxidative stress under *in vitro* conditions. The *in vitro* comet assay results showed that the *Glycyrrhiza glabra* extract protected against H_2O_2 induced cellular DNA damage. These findings indicated promising antioxidant and antigenotoxic potential of *Glycyrrhiza glabra* and need further exploration for translating these findings to its possible health benefits.

Keywords: oxidative stress; DNA damage; genotoxicity.

1. Introduction

Plants have been a major source of medicine since ancient times. The demand for herbal medicines is on the increase across the world since herbs have stood the trial of time for their safety and efficacy [1]. *Glycyrrhiza glabra* Linn. (Family- Fabaceae) is a very well used medicinal plant in traditional medicine systems across the globe. The root and rhizome are the major medicinal parts used. *Glycyrrhiza glabra* is one of the oldest and most frequently used herbs in traditional Chinese medicine. It is an important part of Indian Traditional Medicine Systems. In recent years, many studies have reported that the active compounds isolated from *Glycyrrhiza glabra* possess antitumor, antiviral, antimicrobial, immunoregulatory, anti-inflammatory, etc. [2,3,4].

The present study aims to study the antioxidant potential of ethanolic extract of *Glycyrrhiza glabra* and its potential to protect tissue and cellular DNA from oxidative stress under *in vitro* conditions.

2. Materials and Methods

2.1. Preparation of ethanolic extract.

The plant part used in the present study is the root of *Glycyrrhiza glabra* purchased from the local market. These were ground to get the powder, which was then extracted using ethanol. The concentrated extract was then dried by evaporation, weighed, and stored for further use.

2.2. Phytochemical analysis.

The ethanolic extract of *Glycyrrhiza glabra* was subjected to various qualitative phytochemical screening [5] to test secondary metabolites such as cardiac glycosides and coumarins, quinones, saponins, terpenoids, phlobatannins, steroids, anthraquinones, tannins, phenols, and flavonoids.

2.3. HPLC analysis.

HPLC analysis of the extract was done at KFRI, Thrissur, Kerala. The instrument specifications are as follows. Shimadzu system controller: SCL-10Avp, Shimadzu UV/Vis Photodiode array detector: SPD-M10 Avp, Shimadzu solvent delivery pump: LC 10ATvp, Shimadzu column oven: CTO-10ASvp, HPLC Column Luna 5u C18: 250x4.6mm (Phenomenex), Hamilton microsyringe (25ul capacity), Pump mode: Gradient pump, Sample volume required for analysis: 20ul.

2.4. Antioxidant activity analysis.

The free radical scavenging potential of the ethanolic extract of *Glycyrrhiza glabra* was studied using DPPH free radical scavenging assay and Hydroxyl free radical scavenging assay [6]. The total antioxidant capacity was measured according to Preito *et al*. [7], and the reducing power was determined by the method of Yen and Duh [8].

2.5. Protection of tissue against oxidative stress under in vitro.

Chicken Liver tissue homogenate was exposed to 1 mM H_2O_2 in the presence or absence of different concentrations of ethanolic extract of *Glycyrrhiza glabra* for 10 minutes, and the levels of GSH and extent of lipid peroxidation was analyzed as detailed below. Reduced glutathione was determined according to the method of Moron *et al*., [9] with minor modifications.

100 µL of tissue homogenate was mixed with 63 µL of 25% TCA and cooled on ice for 5 minutes. It was mixed with 300 µL of 5% TCA and then centrifuged at 3000 g for 5 minutes. 150 µL of the supernatant was mixed with 350 µL of sodium phosphate buffer (0.2M, pH 8.0) and 1.0 mL of DTNB (0.6 mM in 0.2M, pH 8.0 phosphate buffer). The yellow color obtained was measured at 412 nm. A standard graph prepared using different concentrations (10-50 nmoles) of GSH was used to calculate the samples' GSH content in nmol/mg protein.

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Buege and Aust [10]. The peroxidation of PUFAs forms malondialdehyde (MDA). MDA is a TBA reacting substance, and the product formed between MDA and TBA can be estimated at 532 nm.

Briefly, the reaction mixture contained 1 mL of tissue homogenate (100 mL of 10 % tissue homogenate + 900 mL distilled water) and 1mL of TBA reagent (0.375% thiobarbituric acid, 0.025 N HCl, 15% trichloroacetic acid and 6.0 mM EDTA). The reaction mixture was heated at 90 ^{o} C for 30 minutes, cooled, and centrifuged at 10,000xg for 10 minutes. The amount of TBARS in the supernatant was obtained by measuring the absorption at 532 nm. The lipid peroxidation values are expressed as n moles of MDA per mg protein.1, 1, 3, 3-tetraethoxypropane was used as the standard.

Protein content in the tissue was determined according to the method of Lowry *et al*. [11]. Protein content was calculated from the standard graph plotted using different concentrations (0-500µg/mL) of bovine serum albumin (BSA).

2.6. Alkaline single cell gel electrophoresis or Comet assay-in vitro.

Alkaline single cell gel electrophoresis was performed [12] under *in vitro* conditions on human peripheral blood leukocytes. The DNA strand breaks were introduced by treatment with 1 mm H_2O_2 in human peripheral blood lymphocytes. The extent of DNA damage was measured using alkaline single cell gel electrophoresis. Blood was incubated with I mM H_2O_2 in the presence or absence of different *Glycyrrhiza glabra* extract (1mg/ml or 2mg/ml) for 5 minutes. After incubation, the samples were analyzed by comet assay. Microscopic slides were coated with normal melting point agarose, and 200 µl of 0.8% low melting point agarose containing 50µl of treated cells (containing 10^4–10^5 cells) were added onto the slide, and the slides were kept at 4 ◦C. After solidification, the slides were immersed in a prechilled lysing solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na2EDTA, pH 10, , 1% TritonX 1% DMSO) and kept for 1h at 4◦C. After lysis, slides were drained and placed in a horizontal electrophoretic apparatus filled electrophoresis buffer (300mM NaOH, 0.2% DMSO, 1mM EDTA, pH≥13). The slides were equilibrated in a buffer for 20 min, and electrophoresis was carried out for 30 min at 20 V, 300 mA. After electrophoresis, the slides were washed gently with 0.4mM Tris–HCl buffer, pH 7.4 followed by distilled water, dried, and silver staining was carried out.

2.7. Statistical analysis.

The results are presented as mean± SD of the studied groups. Microsoft Excel and GraphPad Prism software were used.

3. Results and Discussion

The roots of *Glycyrrhiza glabra* were collected, shade dried, and powdered. The ethanolic extract was obtained by soxhlet extraction. The extract was evaporated to dryness, and the percentage of yield was found to be 29 %.

3.1. Phytochemical analysis.

As can be seen from Table 1, the ethanolic extract of *Glycyrrhiza glabra* contained coumarins, saponins, quinones, cardiac glycosides, terpenoids, phlobatannins, anthraquinones, tannins, phenols, and flavonoids.

Table 1. Phytochemical analysis of ethanolic extract of *Glycyrrhiza glabra*, + indicates the presence and – indicates absence.

Sl.no	Phytochemicals	Ethanolic extract
1	Coumarins	+
2	Saponin	+
3	Quinone	+
4	Cardiac glycosides	+
5	Terpenoids	+
6	Phlobatannins	+
7	Anthraquinones	+
8	Tannins	+
9	Phenols	+
10	Flavanoids	+

The ethanolic extract's phytochemical screening showed that the roots were rich in coumarins, saponins, quinones, cardiac glycosides, terpenoids, phlobatannins, anthraquinones, tannins, phenols, and flavonoids. The HPLC analysis as shown in Figure 1 confirmed the presence of these bioactive constituents in the extract

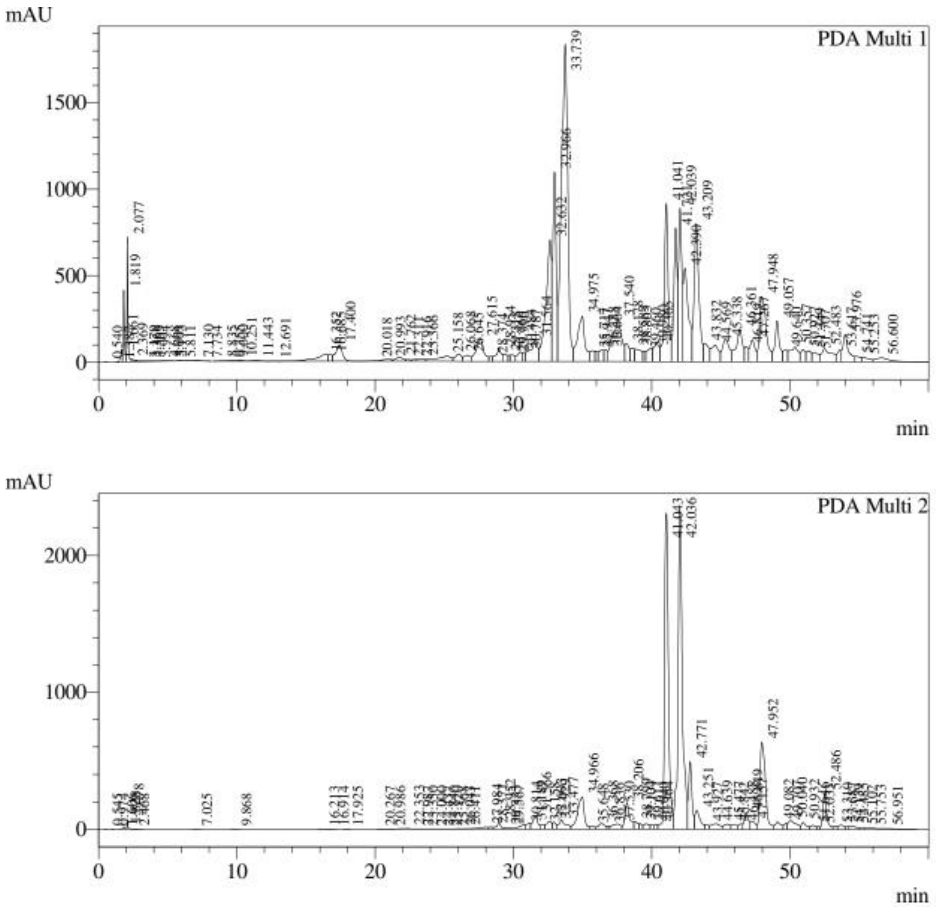

Figure 1. High performance liquid chromatogram of ethanolic extract of *Glycyrrhiza glabra.*

3.2. Analysis of antioxidant activity.

The antioxidant activity of the ethanolic extract of *Glycyrrhiza glabra* was determined using DPPH free radical scavenging assay. Figure 2 gives the percentage of inhibition of DPPH free radicals by different concentrations of the extract. The result indicates that the extract possesses free radical scavenging activity in a dose-dependent manner.

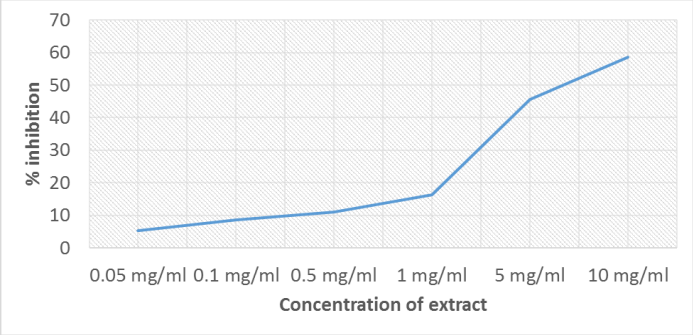

Figure 2. DPPH free radical scavenging activity of ethanolic extract of *Glycyrrhiza glabra.*

The antioxidant activity of the ethanolic extract of *Glycyrrhiza glabra* was determined using the hydroxyl radical scavenging assay. Figure 3 gives the percentage of inhibition of hydroxyl radicals by different concentrations of the extract. The result indicates that the extract possesses significant hydroxyl radical scavenging activity.

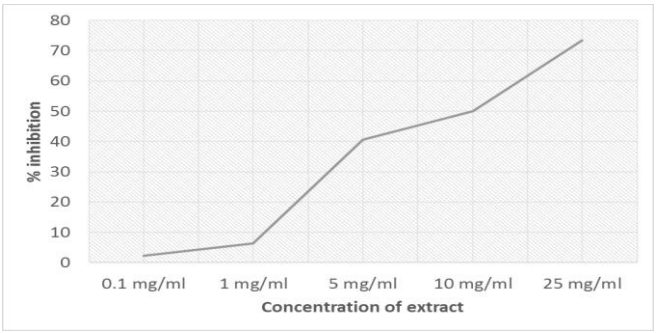

Figure 3. Hyrdoxyl radical scavenging activity of ethanolic extract of *G.glabra.*

The antioxidant activity of ethanolic extract of *Glycyrrhiza glabra* was determined using total antioxidant capacity assay. Figure 4 gives the percentage inhibition by different concentrations of the ethanolic extract.

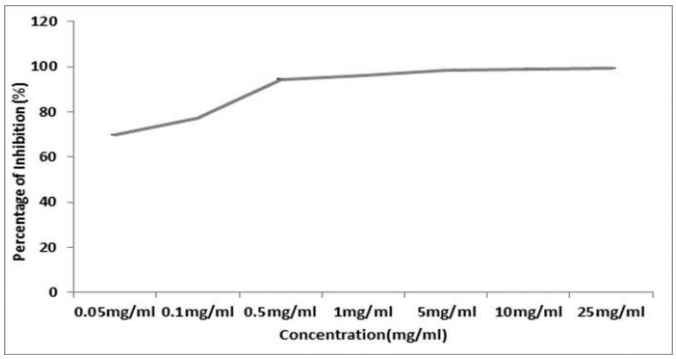

Figure 4. Total antioxidant capacity of ethanolic extract of *G.glabra.*

The antioxidant activity of ethanolic extract of *Glycyrrhiza glabra* was determined using total reducing power assay. Figure 5 gives the percentage inhibition by different concentrations of the ethanolic extract.

3.3. Protection of tissue against oxidative stress in vitro.

H_2O_2 is known for its oxidant activity, and it induces oxidative stress in the cells. To determine whether *Glycyrrhiza glabra* inhibits the H_2O_2 induced cell damage in liver tissue, GSH assay was performed by treating the cells with 1mM H_2O_2.

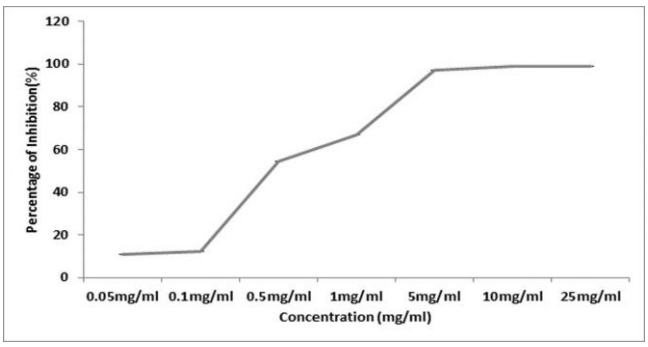

Figure 5. Total reducing power activity of ethanolic extract of *G.glabra.*

The results are presented in Figure 6. A significant reduction in GSH levels was found following exposure to oxidative stress. The GSH content of normal liver tissue and H_2O_2 treated control is 41.34 and 31.83 nanomoles/mg protein. It shows that the activity of GSH in the tissues studied was significantly decreased in the H_2O_2 treated control compared with normal tissue. It was also observed that *Glycyrrhiza glabra* treated cells showed higher glutathione as compared to only H_2O_2 treated cells where decreased glutathione was observed. The presence of *Glycyrrhiza glabra* and H_2O_2 restored the activities of the antioxidant enzymes and the level of glutathione to near normal compared to the corresponding H_2O_2 treated.

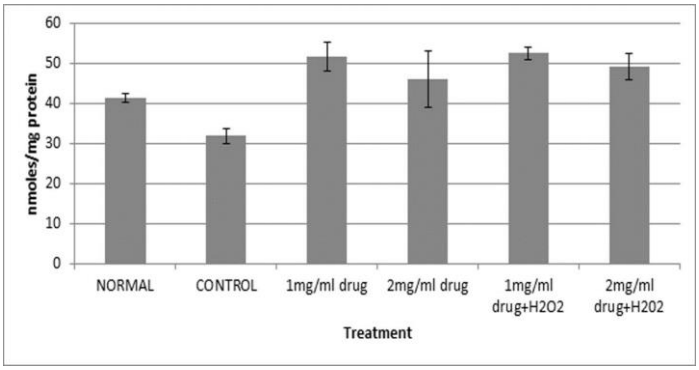

Figure 6. Levels of GSH (nanomoles/mg protein) in liver tissue homogenate treated with 1mM H_2O_2 and different concentrations of *Glycyrrhiza glabra* extract under *in vitro* conditions.

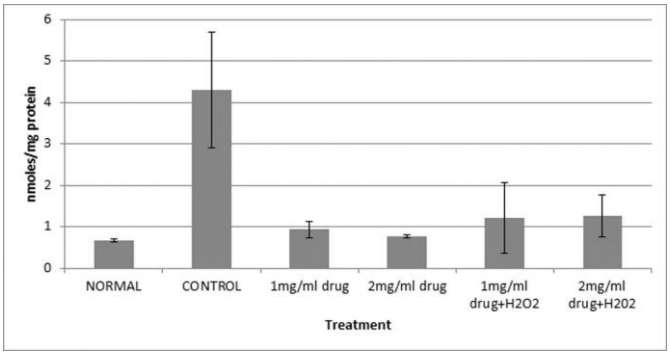

Figure 7. Extent of lipid peroxidation in liver tissue homogenate treated with 1mM H_2O_2 and different concentrations of *Glycyrrhiza glabra* extract under *in vitro* conditions.

MDA is an organic compound that is the end product of the lipid peroxidation of polyunsaturated fatty acids known to induce oxidative stress. To determine whether *Glycyrrhiza glabra* inhibits the H_2O_2 induced cell damage in liver tissue, lipid peroxidation assay was performed by treating the cells with 1Mm H_2O_2, and the results are presented in Figure 7. The Lipid peroxidation content of normal liver tissue and H_2O_2 treated control is obtained as 0.67 and 4.29 nanomoles/mg protein, respectively. The level of lipid peroxidation

in H_2O_2 treated cells in the presence of *Glycyrrhiza glabra* was found to be much lower. The presence of *Glycyrrhiza glabra* and H_2O_2 restored the activities of the antioxidant enzymes and the level of lipid peroxidation to near normal compared to the corresponding H_2O_2 treated cells.

The levels of GSH and Lipid peroxidation indicated the potential of *Glycyrrhiza glabra* to protect tissue against oxidative stress induced by H_2O_2 under *in vitro* conditions.

3.4. Analysis of genoprotective activity.

Comet assay is a very useful tool that provides information regarding the extent of cellular DNA damage [13,14]. Comet assay was performed with human peripheral blood lymphocytes obtained through venipuncture. The DNA strand breaks were introduced by treatment with 1 mM H_2O_2 in human peripheral blood lymphocytes. The extent of DNA damage was measured using alkaline single cell gel electrophoresis. Blood was incubated with I mM H_2O_2 in the presence or absence of different concentrations of *Glycyrrhiza glabra* extract (1mg/ml or 2mg/ml) for 5 minutes. After incubation, the samples were analyzed by comet assay.

In comet assay, cells embedded in agarose on a microscope slide are lysed using detergent and high salt to form nucleoids containing supercoiled loops of DNA and electrophoresed. The slides were observed under a microscope after silver staining.

When the slides were observed by microscopy after the assay, it was observed that:

a) The cells treated with H_2O_2 alone showed a -like comet appearance. The cells had an elongated tail portion, comprised of the broken fragments of the cellular DNA, which migrated out of the nucleus during the electrophoresis. This indicated the extent of DNA damage.

b) The cells treated with *Glycyrrhiza glabra* extract alone remained circular, indicating that the extract by itself was not genotoxic.

c) The cells treated with *Glycyrrhiza glabra* extract along with H_2O_2 appeared as circular discs indicating that the extract offered protection against H_2O_2 induced DNA damage.

The endogenous oxidation processes and exogenous sources produce reactive oxygen species (ROS) and free radicals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have both beneficial and detrimental effects on the living systems. Overproduction of the free radicals induces oxidative stress culminating in biological damage. Oxidative stress caused by free radicals is involved in the onset of cancer, rheumatoid arthritis, liver cirrhosis, arteriosclerosis, and degenerative diseases related to aging [15,16]. Nearly all living organisms have evolved a complicated antioxidant system mainly comprised of antioxidant enzymes which include catalase (CAT), glutathione peroxidize (GSHPx), superoxide dismutase (SOD), or substances including ascorbic acid, a-tocopherol, carotenoids, glutathione, and polyphenol substances to protect the cells and organ systems. However, the production of natural antioxidants under normal physiological conditions is insufficient to prevent all damages caused. Consumption of antioxidant supplements or foods that possess antioxidative activities may reduce oxidative damage [17,18]. Several synthetic antioxidants such as Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertbutylhydroquinone (THBQ), and propyl gallate are in common use nowadays. However, several reports suggested that synthetic antioxidants promote tumor formation [19,20]. In this scenario, natural antioxidants received much attention and are being extensively studied. A wide range of antioxidants has been isolated from plant materials, including cereal crops, herbs, spices, vegetables, fruits, oilseeds, leaves, and roots. It is a known fact that *Glycyrrhiza glabra* is one of the richest sources of biologically active compounds such as phenolic and flavonoid compounds, which act as

primary antioxidants or free radical scavengers [21]. It possess antitumor, antimicrobial, anti-inflammatory, immunoregulatory activities, etc. [22,23,24]. In the present study, the antioxidant and genoprotective activity of ethanolic extract of *Glycyrrhiza glabra* was evaluated by various *in vitro* assays.

Free radical scavenging activity is a potent indicator of the bioactive compounds that can act as an effective phytotherapeutic. The DPPH antioxidant assay, Hydroxyl radical scavenging assay, the reducing power assay, and the total antioxidant capacity showed the potent antioxidant potential of *Glycyrrhiza glabra* extract. *Glycyrrhiza glabra* extract prevented oxidative damage, as shown by the level of GSH and lipid peroxidation in chicken liver tissue treated with 1 mM H_2O_2. The presence of *Glycyrrhiza glabra* along with H_2O_2 restored the level of glutathione and lipid peroxidation to near-normal levels. H_2O_2 induced DNA strand breaks in the peripheral blood lymphocytes were measured using alkaline single cell gel electrophoresis. The results revealed that *Glycyrrhiza glabra* extract offered protection against H_2O_2 induced cellular DNA damage.

4. Conclusions

Glycyrrhiza glabra extract could ameliorate H_2O_2 induced oxidative damage and cellular DNA damage under *in vitro* conditions, which may be due to its antioxidant and free radical scavenging properties. These findings indicated promising antioxidant and antigenotoxic potential of *Glycyrrhiza glabra* and need further exploration for translating these findings to its possible health benefits.

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Conflicts of Interest

The authors declare no conflict of interest.

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